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14. ABSTRACT Resuscitation from hemorrhagic shock with fluid (Ringer's Lactate) and blood may not correct all of the metabolic abnormalities associated with shock. Patients who are apparently fully resuscitated may yet develop organ failure syndromes such as respiratory failure and renal failure. The objective of our research has been to evaluate the therapeutic efficacy of three pharmacological agents which have been suggested as adjuncts to standard resuscitation (glutamine, crocetin, and dehydroepiandrosterone (DHEA)) by studying their use in a rodent model. The emphasis throughout was to assess the potential of these agents for treating battlefield injuries. Both glutamine and crocetin produced much more rapid recovery of depleted ATP levels after shock and resuscitation, decreased apoptotic mediators and apoptosis itself, and improved survival. DHEA did not improve hemodynamic response in a porcine shock model, nor did it affect ATP or apoptosis. Glutamine and crocetin are good candidates for clinical trials.					
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Dr. Charles W. Van Way
charles.vanway@tmcmcd.org
School of Medicine, Univ. Missouri-Kansas City
Surgery, Shock/Trauma Research Center
2411 Holmes St.
Kansas City, MO
64108
Phone: (816) 404-5364
Fax: (816) 404-5363

Website URL: none

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Introduction

Resuscitation from hemorrhagic shock has largely been limited to the use of fluid (Ringer's Lactate) and blood. But such resuscitation clearly fails to correct all of the metabolic and biochemical abnormalities associated with shock. Patients who have been apparently fully resuscitated may nonetheless go on to develop respiratory failure, renal failure, or other organ failure syndromes. The objective of our research has been to evaluate the therapeutic efficacy of three pharmacological agents which have been suggested as additives to standard resuscitation following hemorrhagic shock. These agents were glutamine, crocetin, and dehydroepiandrosterone (DHEA). Glutamine is a nitrogen donor in nucleotide synthesis and a cellular energy substrate, and has previously been shown by us to improve recovery of ATP and ADP levels. Crocetin is a carotenoid shown in other studies to improve tissue oxygenation. DHEA is a hormone, which has been shown by others to improve certain aspects of cellular metabolism. In our investigations, we concentrated on measuring the recovery of cellular energy levels, lowering the level of nucleotide metabolites, diminishing the amount of late apoptosis, altering the hemodynamic response, and improving survival. With glutamine and crocetin, our studies were done in the rodent (rat) model of shock and resuscitation. For DHEA, which has been more extensively studied by others, our studies were done in the porcine model, to better assess the potential of this agent for clinical use. The emphasis throughout was to assess the potential of these three agents for clinical use in treatment of battlefield injuries.

To briefly summarize our findings, both glutamine and crocetin were associated with a much more rapid recovery of depleted ATP levels after shock and resuscitation, and produced a decrease in apoptotic mediators and apoptosis itself at 24 and 48 hours after shock. With both agents, there was an increase in survival. On the other hand, DHEA did not improve the hemodynamic response in the porcine shock model. It had little effect on tissue adenosine nucleotide levels or on the apoptotic mediators. Because

the hemodynamic results and the preliminary biochemical results showed little benefit, we did not study this compound further. Glutamine and crocetin may prove to be good candidates for clinical trials.

Aims

After shock and resuscitation, the major tissue adenosine nucleotides, ATP and ADP, are depleted to around 30% to 40% of baseline levels. There are increased levels of AMP and the nucleotide metabolites adenosine, inosine, hypoxanthine, and xanthine. Using a sub-lethal shock model, we have determined that ATP and ADP remain depressed in the rat for up to 48 hours after shock. There is increased apoptosis in the liver at 24 and 48 hours, as compared to sham-operated controls. The extent of apoptosis correlates on the one hand with energy depletion, and on the other with levels of intermediaries in the apoptosis pathway, notably caspase-3, and with evidence of mitochondrial damage as indicated by cytosolic cytochrome-c. Levels of bcl-2, a protective mitochondrial membrane protein, were found to be depressed after shock and resuscitation. Our approach has been to study these post-shock events following the administration of glutamine, crocetin, and DHEA as a component of a resuscitation regimen which includes administration of lactated Ringer's solution and return of shed blood. It has been our central hypothesis that if the changes in ATP and ADP can be corrected quickly during resuscitation, then the mediator response will be blunted, and late tissue damage minimized, and survival will be enhanced.

Results

Studies on glutamine, crocetin, and DHEA have been largely completed. Both crocetin and glutamine have been shown to accelerate restoration on cellular ATP levels following shock and resuscitation, decrease the amount of apoptosis at 24 and 48 hours, decrease caspase-3 and cytosolic cytochrome-c, restore bcl-2 level to normal, and significantly improved survival (See also the data in appendices 1-3). Studies of the use of DHEA in the large animal model (porcine) showed it to have little affect on the hemodynamic response (Appendix 4). Studies of the tissue adenosine nucleotides and apoptosis showed that DHEA has little or no affect. In this study, we used 836 male Sprague-Dawley Rats (10-12 weeks old) and 44 male Yorkshire pigs (44-66 lbs). All experiments on animals were carried out on pain level C.

Studies at the level of gene activation have shown that crocetin treatment markedly affects the level of mRNA for inflammatory cytokines TNF- α and IL-1 β and for iNOS following shock and resuscitation. (Appendix 5). This indicates that the agent has an effect at a very basic level in the cell. Further investigations are under way to determine the mechanisms by which this compound has such an effect.

In other subsequent studies focused on glutamine and crocetin, we have found that both of these agents appear to modulate cytokine response in two different models of septic shock, the cecal ligation and puncture model (rat), and LPS stimulation of cells.

For this latter, we have investigated both mouse peritoneal macrophages and RAW cells. The initial findings from these studies are currently being prepared for publication. However, we regard them as highly important. First, both agents may be useful in therapy of sepsis. Second, the observation that these agents are effective in both hemorrhagic shock and sepsis indicates that these two may have more in common than has generally been believed. More specifically, stimulation of the acute inflammatory reaction occurs in both, and now appears to be carried out through very similar mechanisms in these two conditions.

Peer Reviewed Journal Articles

1. Van Way C.W., Dhar A. and Morrison D.C. Cellular Energetics in Hemorrhagic Shock: Restoring Adenosine Triphosphate to the cells. J. Trauma Injury, Infection and Critical Care 2003; 54:S169-176.
2. Dhar A, Kujath D, Van Way C.W. Glutamine Administration During Total Parenteral Nutrition Protects Liver Adenosine Nucleotides During and After Subsequent Hemorrhagic Shock. J Parent Ent Nutr 2003; 27:246-251.
3. Van Way C.W., Dhar A., Morrison D.C. Hemorrhagic Shock: A New Look at an Old Problem. Missouri Medicine, 2003; 100:518-523.
4. Helling, T. The Liver and Hemorrhagic Shock. J Am Coll Surg 2005; 201:774-783.

Submitted or in preparation but not yet published:

5. Yang R, Tibbs BM, Chang B, Nguyen C, Woodall C, Steppacher R, Van Way CW III, Morrison DM. Effect Of DHEA On The Hemodynamic Response To Resuscitation In A Porcine Model Of Hemorrhagic Shock.
6. Yang R, Thomas AM, Tan X, Qureshi N, Morrison DC, Van Way CW III, Crocetin Enhances Survival In Cecal Ligation And Puncture With Ligation Of The Cecal Artery
7. Yang R, Thomas Am, Tan X, Qureshi N, Shen, J, Morrison Dc, Van Way Cw III, Crocetin Inhibits Expression Of Inflammation-Related Genes And Inos In Hemorrhagic Shock
8. Thomas AM, Tan X, De Sousa J, Yang, R, Qureshi N, Van Way CW III. Effects of Glutamine, Crocetin and Amino Acids on Proteasomal activity and on Cytokine Production by RE-LPS-Stimulated Macrophages.

Abstracts

1. American Society for Parenteral and Enteral Nutrition Clinical Congress, January 21-23, 2001. (Chicago, IL) The Effect of Glutamine given during Resuscitation from Hemorrhagic Shock upon Adenosine Nucleotides in Small Bowel Mucosa. (Presented by BT Moore)
2. Society of Critical Care Medicine, February, 2001. The Effect of Crocetin on Cellular Damage following Hemorrhagic Shock. J. Crit. Care. Med. A101, 2001^{17th} International Union of Biochemistry and Molecular Biology and Annual Meeting of American Society of Biochemistry and Molecular Biology, 1997. Possible Mechanism of Tissue Damage during Hemorrhagic Shock. FASEB J., A1519, 1997.
3. Society of Critical Care Medicine, February, 2001. Prevention of Late Cellular Injury by Glutamine Resuscitation after Hemorrhagic Shock. J. Crit. Care Med. 29, A13, 2001
4. Missouri State Medical Association, April 5-8, 2001. (St. Louis, MO) Van Way CW III. Restoring Cellular Energy in Hemorrhagic Shock. (Presentation)
5. Combat Fluid Resuscitation 2001, June 16-18, 2001. (Bethesda, MD) Van Way CW III. Cellular Energetics in Hemorrhagic Shock. (Presentation)
6. The Fourth Annual Steven R. Hall, MD, Trauma Symposium, September 27-29, 2001. (Ridgedale, MO) Van Way CW III. New Concepts in Hemorrhagic Shock - Cellular Energetics, and Nutrition and Injury - Meeting metabolic needs. (Presentation)
7. University of Nebraska, January 17-18, 2002. (Omaha, NE) Van Way CW III. The Continuing Mystery of Hemorrhagic Shock
8. Shock Society, June, 2002 (Big Sky, MT) Dhar A, Berghoff K., Maxfield D., Landis M., Gao JJ, Morrison, D C, Qureshi N., Ray BK, Ray A, Van Way C. Promotion of Apoptosis by Changes in NF- κ B and Serum Amyloid A Factor after Hemorrhage and Resuscitation. Shock, 17, A39, 2002. (Poster)
9. European Society for Parenteral and Enteral Nutrition (ESPEN), Aug 31-Sep 5, 2002. (Glasgow, Scotland, UK) Van Way CW III. Glutamine Administration Prevents Liver Apoptosis after Hemorrhagic Shock. (Presentation)
10. Advanced Technology Applications for Combat Casualty Care (ATACCC), Sep 9 – Sep 11, 2002. (St. Pete Beach, FL) Van Way CW III Therapeutic Intervention in Treatment of Experimental Hemorrhagic Shock. (Presentation)
11. Missouri Chapter, American College of Surgeons, June 2003. (Lake Ozark, MO) Martin LR, Dhar A, Yang R, Woodall C, Iqbal C, Morrison DC, Van Way CW. Administration of Glutamine During Resuscitation After Hemorrhagic Shock Decreases Liver Damage. (Presented by L Martin)

12. Missouri Chapter, American College of Surgeons, June 2003. (Lake Ozark, MO) Nguyen C, Yang R, Dhar A, Tibbs B, Resch G, Helling T, Van Way CW. Effect of DHEA on Continuous Cardiac Output and Blood Pressure in a Porcine Model of Hemorrhagic Shock. (Presented by C Nguyen)
13. Shock Society, June 7-11, 2003 (Phoenix, AZ) Morrison DC, Tan X, Qureshi N, Dhar A, Van Way CW, Gao JJ. Bacterial Lipopolisaccharide Suppresses Expression of DNA Methyltransferase I in Mouse Macrophages. (Poster) Shock, 2003. 19 (Suppl 1): 19.
14. Shock Society, June 7-11, 2003. (Phoenix, AZ) Dhar A, Yang R, Gao JJ, Morrison DC, Qureshi N, Ray BK, Ray A, Van Way CW. Role of Inflammatory Transcription Factors on Apoptosis and Caspase-7 Expression Following Hemorrhagic Shock. Shock, 2003. (Poster) 19(Suppl 1): 47.
15. Shock Society, June 7-11, 2003. (Phoenix, AZ) Martin LR, Dhar A, Yang R, Nguyen C, Chang B, Woodall C, Morrison DC, Van Way CW. Glutamine Administration During Resuscitation After Hemorrhagic Shock Improves Survival and Decreases Cellular Injury. Shock, 2003 (Poster) 19(Suppl 1): 52.
16. Kansas City Area Life Sciences Institute, March 27, 2003 (Kansas City). Van Way CW III. Therapeutic Intervention in Treatment of Experimental Hemorrhagic Shock. (Presentation)
17. Shock Society, June 7-11, 2003. (Phoenix, AZ) Yang R, Chang B, Iqbal C, Tibbs B, Resch G, Helling T, Dhar A, Van Way CW. Effect of DHEA on Continuous Cardiac Output and Blood Pressure in a Porcine Model of Hemorrhagic Shock and Resuscitation. Shock, 2003. (Poster) 19(Suppl 1): 59.
18. Shock Society, June 7-11, 2003. (Phoenix, AZ) Dhar A, Yang R, Gao JJ, Morrison DC, Qureshi N, Ray BK, Ray A, Van Way CW III. Role of Inflammatory Transcription Factors on Apoptosis and Caspase 7 Expression Following Hemorrhagic Shock. Shock, 2003. (Poster) 19(Suppl 1): 47.
19. Shock Society, June 7-11, 2003. (Phoenix, AZ) Morrison DC, Tan X, Qureshi N, Dhar A, Van Way CW III, Gao JJ. Bacterial Lipopolisaccharide Suppresses Expression of DNA Methyltransferase I in Mouse Macrophages.(Poster) Shock, 2003. 19(Suppl 1):19.
20. Missouri Chapter, American College of Surgeons, June 2003. (Lake Ozark, MO) Martin LR, Dhar A, Yang R, Woodall, C Iqbal C, Morrison DC, Van Way CW III. Administration of Glutamine During Resuscitation After Hemorrhagic Shock Decreases Liver Damage. (Presented by L. Martin)
21. Missouri Chapter, American College of Surgeons, June 2003. (Lake Ozark, MO) Nguyen C, Yang R, Dhar A, Tibbs B, Resch G, Helling T, Van Way CW III. Effect of DHEA on Continuous Cardiac Output and Blood Pressure in a Porcine Model of Hemorrhagic Shock. (Presented by C Nguyen).

22. Shock Society, June 5-8, 2004 (Halifax, Nova Scotia, Canada) Thomas A, Yang R, Nguyen C, Chang W, Dhar A, Van Way CW III. Effect of Treatment with DHEA in a Murine Model of Hemorrhagic Shock.
23. Shock Society, June 5-8, 2004 (Halifax, Nova Scotia, Canada) Yang R, Steppacher R, Nguyen C, Chang W, Thomas A, Dhar A, Van Way CW III. Nucleotide Changes after DHEA Treatment in a Porcine Model of Hemorrhagic Shock.

Awards

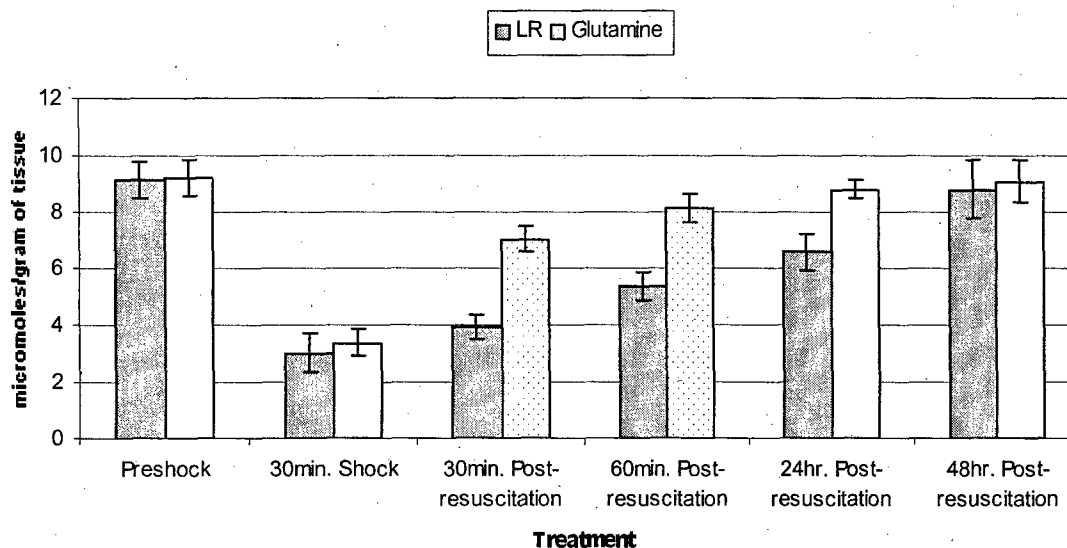
1. Missouri Chapter, American College of Surgeons, June 2003 (Lake Ozark, MO) Martin LR, Van Way CW, et al Administration of Glutamine During Resuscitation After Hemorrhagic Shock Decreases Liver Damage. Dr. L. Martin won third prize in Resident Competition. This paper was presented in the Region IX Committee on Trauma competition in December, 2003, where won first prize. At the National COT meeting, it was first runner-up.
2. UMKC Research Day, April 8, 2003. Administration of Glutamine during Resuscitation after Hemorrhagic Shock Decreases Cellular Damage. Iqbal C., Dhar A., Martin L.R., Yang R., Woodall C., Morrison D.C., and Van Way C.W. Mr. Corey Iqbal, a Year 6 Medical Student, won UMKC Medical School Alumni award for his presentation.
3. UMKC Research Day, April 8, 2003. Crocetin Improves Survival and Cellular Damage Following Hemorrhagic Shock. Nguyen G., Dhar A., Wittek B., Morrison D.C., and Van Way C.W. Dr. G. Nguyen, a Year 5 Medical Student, won an award for Best Research Paper.

Next Steps And Additional Projects

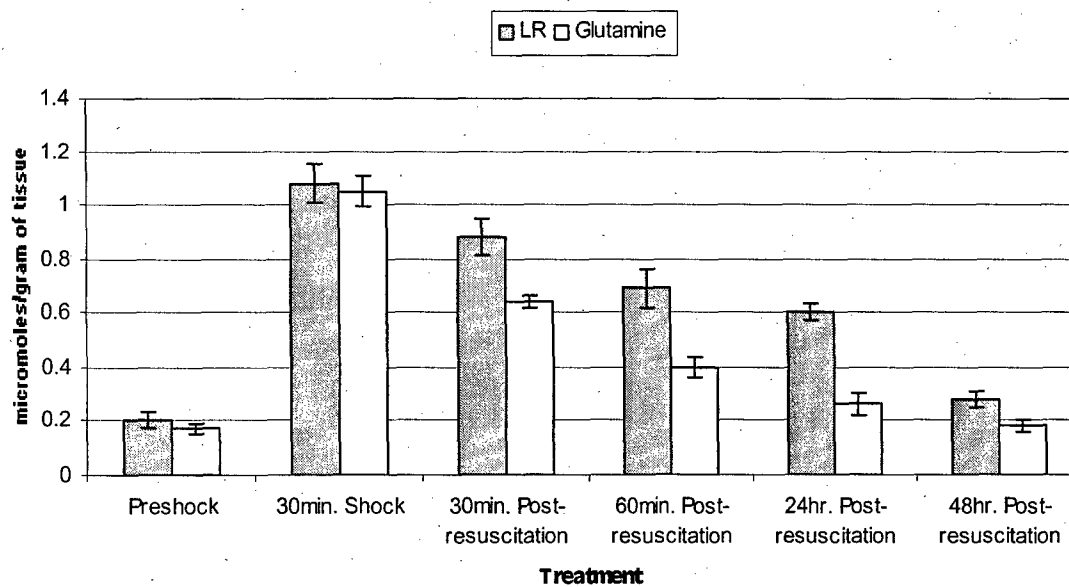
1. There are several studies being carried out to continue to analyze and treat hemorrhagic shock. Currently, we are working on a pilot study to examine the effects of omega-3 fatty acid supplementation on the response to hemorrhagic shock and resuscitation. The goal of this study is to see if supplementation with these compounds for 4 weeks might help them to better respond to hemorrhagic shock.
2. Other studies have been carried out to show that inflammation increases during shock and even more during resuscitation. It appears that crocetin aids in reducing cytokine expression and inflammation after shock and resuscitation (Appendix 6).
3. We are also undergoing projects to determine the role of NF- κ B and the proteasome during hemorrhagic shock. It has been found that an increase in proteasomal activity can be linked to LPS stimulation and in return the increase in cytokine production. Our goal here is to see if hemorrhagic shock stimulates proteasomal activity and NF- κ B activation as well.
4. Efforts to further develop crocetin and glutamine as therapeutic agents are continuing. Glutamine is somewhat difficult to use, as it is not very soluble and is somewhat unstable in solution over the long term. The use of the glutamine-alanine dipeptide, which is cleaved in the blood stream by proteases to glutamine and alanine, is being investigated as a more useful way of delivering glutamine. As for crocetin, its use as a therapeutic agent may be expanded by giving it through the GI tract, thus opening up the possibility that it may be administered orally by first responders.
5. A proposal has been submitted to further study the effect of these compounds on the genomic response to shock and injury by using microarray technology, both in animal models and in humans. This approach would not only provide a very large amount of information about the response to shock and the effect of these particular agents, but the use of microarray studies on the genetic response in the peripheral white blood cells would make it much safer and more productive to carry out such studies in patients. Once the response has been characterized in animal models and validated in patients, new agents can be used in relatively small numbers of patients in order to precisely determine their effect at a very basic level.

APPENDIX 1. Effect of treatment with glutamine during resuscitation upon hepatic ATP levels and upon levels of xanthine, an ATP metabolite. The shock period was 30 minutes long.

ATP Levels After 30 Minutes of Shock

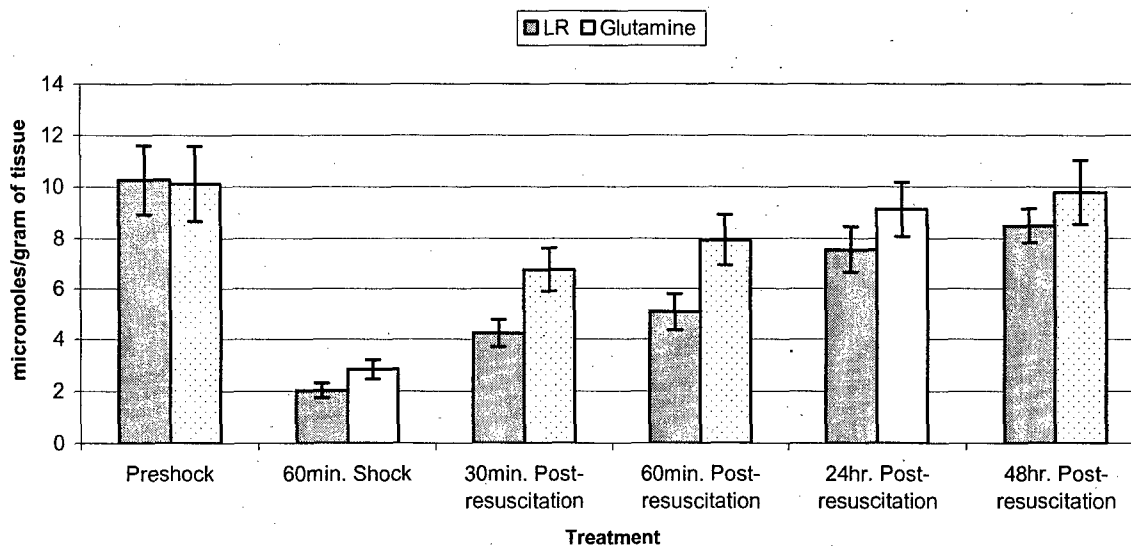


Xanthine Levels After 30 Minutes of Shock

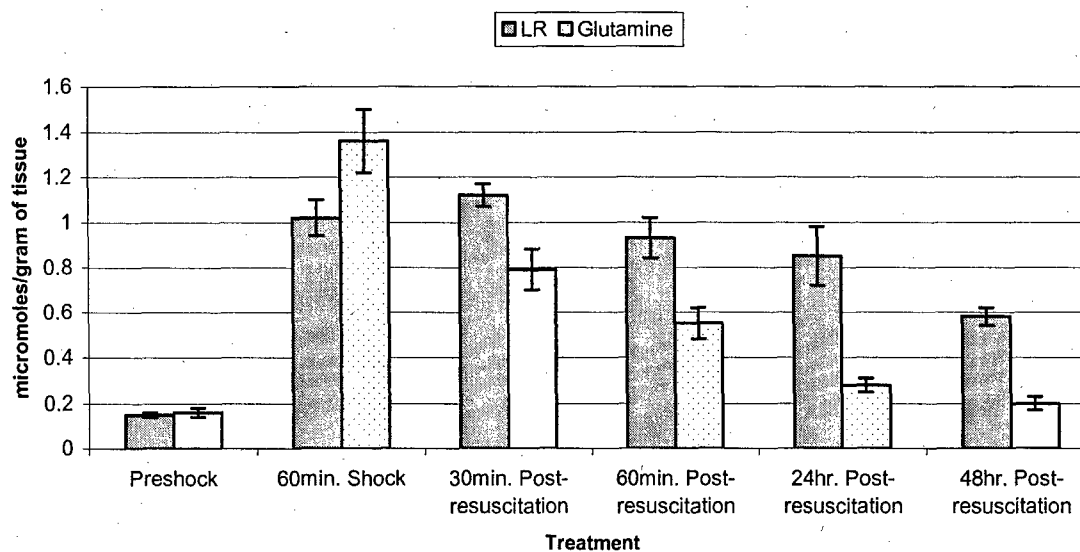


APPENDIX 2. Effect of treatment with glutamine during resuscitation upon hepatic ATP levels and upon levels of xanthine, an ATP metabolite. The shock period was 60 minutes long.

ATP Levels After 60 Minutes of Shock



Xanthine Levels After 60 Minutes of Shock

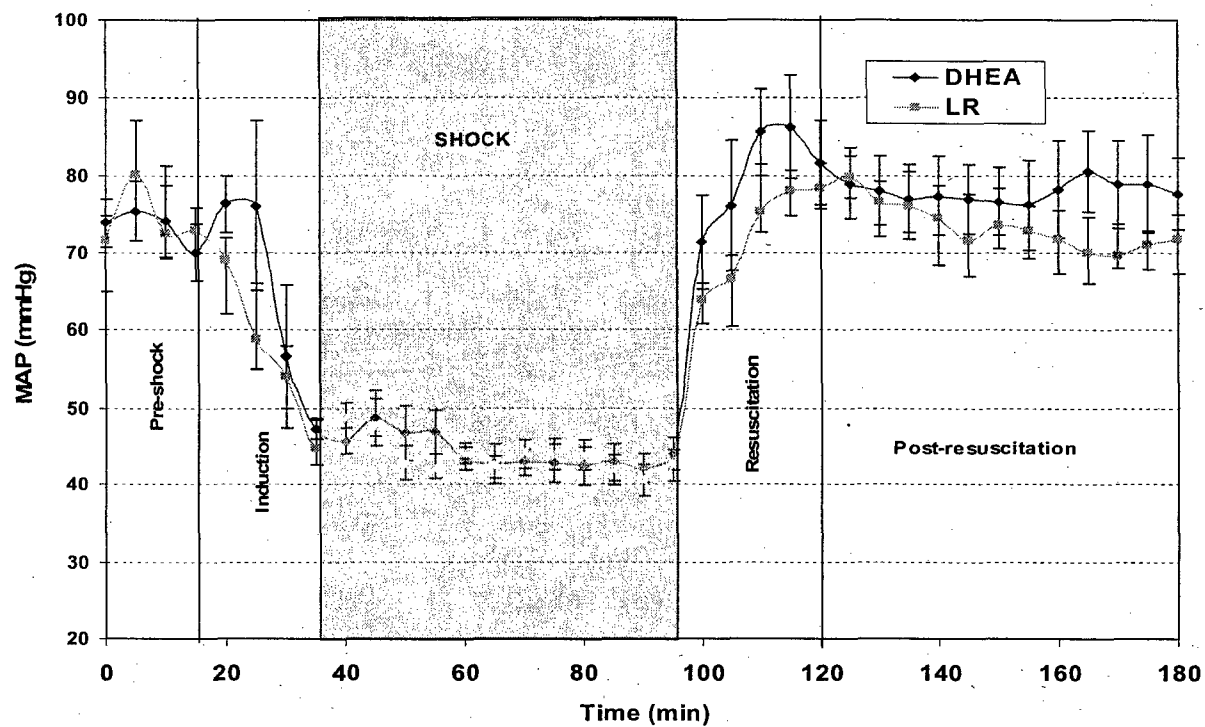


APPENDIX 3: The effect of glutamine treatment during resuscitation upon bcl-2 protein, caspase-3, cytosolic cytochrome c, and apoptosis at 24 and 48 hours post-shock and resuscitation. Both groups resuscitated with lactated Ringer's and with return of shed blood; the glutamine group received glutamine with the lactated Ringer's. The glutamine group showed higher levels of bcl-2 protein, lower caspase-3, lower cytosolic cytochrome c, and less apoptosis than the control group.

bcl-2 ELISA 30 min Shock	LR	SEM	Glutamine	SEM
Pre-shock	125	32	130	20
24 hr PR	52	9	80	7
48 hr PR	39	5	95	12
Casp-3 ELISA 30 min shock	LR	SEM	Glutamine	SEM
Pre-shock	0.12	0.02	0.1	0.03
24 hr PR	0.41	0.09	0.16	0.05
48 hr PR	0.62	0.08	0.2	0.03
Cyt-c ELISA 30 min shock	LR	SEM	Glutamine	SEM
Pre-shock	0.25	0.03	0.28	0.03
24 hr PR	0.65	0.06	0.42	0.03
48 hr PR	0.85	0.07	0.53	0.06
Apoptosis ELISA 30 min Shock	LR	SEM	Glutamine	SEM
Pre-shock	1.82	0.24	1.2	0.28
24 hr PR	3.26	0.46	1.48	0.17
48 hr PR	6.29	0.65	1.31	0.23

Appendix 4: Comparison of pigs treated with normal resuscitation with those treated with normal resuscitation plus DHEA. No difference was seen.

Pigs Shock Model -- MAP



Appendix 5: Percentage of animals (n=9) expressing mRNA for cytokines and iNOS. The group (n=9) treated with crocetin showed markedly less expression of mRNA for inflammatory cytokines and for iNOS than did the control group, which otherwise received the same resuscitation.

(The normal group received anesthesia only. The sham group had vessel cannulation, but was not shocked. The shock group was shocked, but not resuscitated. Both the LR group and the crocetin group were resuscitated with lactated Ringer's and return of shed blood. The crocetin group received 2 mg/kg crocetin at the beginning of resuscitation, while the LR group received an equivalent volume of saline.)

